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# Soluble Endoglin Modulates the Pro-Inflammatory Mediators NF-κB and IL-6 in Cultured Human Endothelial Cells

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**Short title: Soluble Endoglin Modulates Pro-Inflammatory Mediators**

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**ABSTRACT**

Aims: Endoglin is a transmembrane glycoprotein, that plays an important role in regulating endothelium. Proteolytic cleavage of membrane endoglin releases soluble endoglin (sEng), whose increased plasma levels have been detected in diseases related to the cardiovascular system. It was proposed that sEng might damage vascular endothelium, but detailed information about the potential mechanisms involved is not available. Thus, we hypothesized that sEng contributes to endothelial dysfunction, leading to a pro-inflammatory phenotype by a possible modulation of the TGF-β and/or inflammatory pathways.

Main methods: Human umbilical vein endothelial cells (HUVECs) and Human embryonic kidney cell line (HEK293T) were treated with different sEng concentration and time in order to reveal possible effect on biomarkers of inflammation and TGF-β signaling. IL6 and NFκB reporter luciferase assays, quantitative real-time PCR analysis, Western blot analysis and immunofluorescence flow cytometry were used.

Key findings: sEng treatment results in activation of NF-κB/IL-6 expression, increased expression of membrane endoglin and reduced expression of Id-1. On the other hand, no significant effects on other markers of endothelial dysfunction and inflammation, including eNOS, peNOS $^{S1177}$, VCAM-1, COX-1, COX-2 and ICAM-1 were detected.

Significance: As a conclusion, sEng treatment resulted in an activation of NF-κB, IL-6, suggesting activation of pro-inflammatory phenotype in endothelial cells. The precise mechanism of this activation and its consequence remains to be elucidated. A combined treatment of sEng with other cardiovascular risk factors will be necessary in order to reveal whether sEng is not only a biomarker of cardiovascular diseases, but also a protagonist of endothelial dysfunction.

**Key words:** Soluble endoglin; Inflammation; NF-κB; IL-6; Endothelial cells

## INTRODUCTION

Membrane endoglin (CD105, Eng) is an accessory type III receptor for the transforming growth factor-β (TGF-β) superfamily of cytokines [1,2]. Membrane endoglin is a dimer composed of 95-kDa disulfide-linked subunits [3] and involves an extracellular domain of 561 amino acid residues, a single transmembrane domain of 25 amino acid residues, and a short intracellular domain of 47 amino acids that does not include a signal transduction domain. The cytosolic domain of endoglin can be targeted by serine and threonine kinases, including the TGF-β type I and II receptors [4]. Endoglin expression is restricted primarily to cells found in the vessel wall, including endothelial cells (ECs), monocyte/macrophages, as well as to various mesenchymal cells such as fibroblasts and vascular smooth muscle cells (SMCs) [5]. In addition, it was demonstrated to play an important role in endothelial physiology/dysfunction and potentially in atherosclerosis [6].

The membrane bound form of endoglin can be cleaved at its juxtamembrane region by the matrix metalloproteinase 14 (MMP14), releasing a soluble form of endoglin (sEng) into the circulation [7]. Increased plasma levels of sEng have been detected in various pathological conditions related to the cardiovascular system [8], including atherosclerosis [6], type II diabetes mellitus [9] and preeclampsia, where sEng levels correlate with disease severity [10], suggesting it might be a useful biomarker of these diseases progression. Furthermore, several authors have postulated that sEng might induce damage to the vascular endothelium, a topic that we have reviewed recently [1].

Membrane endoglin can bind TGF-β1 and TGF-β3 isoforms in association with TGF-β receptors II and I (ALKs) and form a functional receptor complex [11]. Moreover, *in vitro* studies demonstrated that membrane endoglin affects TGF-β signaling by modulating the activity of Smad transcription factors [12,13]. Several studies have revealed an interplay between two signaling pathways involving membrane endoglin. Membrane endoglin/ALK-

1/Smad1/5 signaling has been shown to stimulate endothelial cell migration, proliferation and tube formation resulting in increased angiogenesis [14]. On the contrary, activation of the membrane endoglin/ALK-5/Smad2 pathway inhibits the activity of endothelial cells, blocks angiogenesis by inhibition of EC proliferation, tube formation and migration, and keeps endothelium quiescent [15,16]. Indeed it was proposed that also soluble endoglin modulates TGF-β family members and their downstream signaling pathways, including TGFβRII/ALK-1/Smad1/5/ld1 pathway [17] and/or TGFβRII/ALK-5/Smad2/3/PAI-1 route [11].

Membrane endoglin is strongly related to the expression and activity of endothelial NO synthase (eNOS) [18], a key enzyme for the proper function of the vascular endothelium [19]. Indeed, altered expression of eNOS or eNOS phosphorylated on serine 1177 (peNOS$^{S1177}$) is involved in endothelial dysfunction and atherogenesis [20]. On the other hand, membrane endoglin was proposed to participate in inflammation by promoting leukocyte adhesion and transmigration [21]. Inflammation is a general key process in the development of endothelial dysfunction. Increased expression of cell adhesion molecules such as vascular cell adhesion molecule (VCAM-1), intercellular adhesion molecule (ICAM-1), monocyte chemoattractant protein-1 (MCP-1) [22], cyclooxygenase-1 (COX-1), or cyclooxygenase-2 COX-2 [23], represents a crucial step for the development of endothelial inflammation/dysfunction. The activated expression of the pro-inflammatory molecules VCAM-1 and ICAM-1 is the ultimate consequence of NF-κB-dependent signaling [24,25]. The nuclear factor NF-kappaB (NF-κB) is a transcription factor involved in many biological processes such as inflammation, cell growth, differentiation, immunity and apoptosis [26,27]. Moreover, TGF-β was shown to affect NF-κB expression and signaling [28,29]. NF-κB cooperates with many other signaling molecules, pathways and transcription factors, such as Signal transducer and activator of transcription 3 (STAT3) [26] and it is an important regulator for IL-6 gene, which has putative binding sites for NF-κB [30]. IL-6 is a

multifunctional cytokine that plays a central role in inflammation and immune response [31]. Circulating IL-6 is produced by activated inflammatory cells within the vessel wall. Thus, NF-κB/IL-6 signaling seems to be important to trigger-vascular inflammation [24].

Although sEng has been proposed to participate in the development of endothelial dysfunction [1], detailed information about the potential mechanisms involved is not available. Thus, we hypothesized that sEng contributes to endothelial dysfunction, leading to a pro-inflammatory phenotype by a possible modulation of the TGF-β signaling pathway. Therefore, we focused on the effects of sEng treatment on endothelial dysfunction markers, biomarkers of inflammation and TGF-β signaling in cultured human endothelial cells.

## MATERIALS AND METHODS

### Cell culture

Human umbilical vein endothelial cells (HUVECs) are primary cells isolated from pooled donors and purchased from Lonza (Basel, Switzerland). HUVECs were grown in EBM-2 medium containing 10% fetal bovine serum (FBS) (Sigma-Aldrich, Czech Republic) and supplemented with EGM-2 SingleQuots (Lonza, France) at 37°C and 5% $CO_2$. In all experiments, HUVECs were cultured in 0.1% gelatin-coated (Sigma-Aldrich) Petri dishes. All experiments were performed with HUVECs at passages between 4 and 9. For starving conditions, HUVECs were incubated for 24 hours in the same medium, but containing 0% of FBS. Gelatin helps HUVECs to attach and grow as it acts as a extracellular matrix. In addition, culturing the cells in the presence of low serum concentration for a short period of time is a standard method to make cells more responsive to soluble factors.

The human embryonic kidney cell line HEK293T was cultured in Dulbecco´s modified Eagle´s medium (DMEM) (Gibco, Massachusetts, USA) and supplemented with 10% FBS (Gibco), 100U/mL penicillin and 100U/mL streptomycin (Gibco) at 37ºC and 5%

$CO_2$. Recombinant human endoglin and TNFα were purchased from R&D Systems (Minnesota, USA). The sEng and TNFα stock solutions of 250 µg/mL and 10 µg/mL respectively were prepared in sterile PBS containing 0.1% bovine serum albumin (Biosera, France) and aliquots were stored at -20°C. All the treatments were done with medium containing 0-2%–FBS and selected concentrations of sEng (40 ng/mL and 500 ng/mL). Concentrations of sEng used in the manuscript were selected within the range of the concentrations found in women with preeclampsia and in a mouse model of preeclampsia that over-expresses soluble human endoglin [32-34]. 2 hours treatment was used for the evaluation of phosphorylation of Smads and $eNOS^{S1177}$. 16 hours treatment were used for the evaluation of changes of all genes and endoglin, VCAM-1, Id-1 and COX-2 expression on protein level. 24 hours treatment was used for the evaluation of IL-6 protein levels in cultured medium, and for Luciferase reporter activity assays of IL-6 and NF-κB. 48 hours soluble endoglin treatment was used for the NF-κB expression.

**Luciferase reporter activity of different promoter constructions**

The IL6 reporter vector encodes the luciferase driven by the IL6 promoter. The NFκB reporter encodes the luciferase driven by a regulatory sequence containing consensus motifs for NFκB binding and therefore is responsive to NFκB activation. The plasmid pKBF-Luc/NF-κB contains three repeats of the NF-κB consensus elements present in the H-2Kb gene upstream of the herpes simplex thymidine kinase gene [35]. The pGL3-IL-6-Luc reporter construct driven by a 651-bp promoter fragment of human IL-6 [36] was kindly provided by Dr. Manuel Fresno (Centro de Biología Molecular Severo Ochoa (CSIC-UAM), Cantoblanco, Madrid, Spain). Transient co-transfection of HEK293T cells was conducted using Lipofectamine 2000 (ThermoFisher Scientific, California, USA) adding 0.8 µg of the pKBF promoter luciferase vector or pGL3-IL6-Luc reporter vector plus 50 ng of pCMV-βgal

vector (Clontech, France) to normalize for transfection efficiency, according to the manufacturer´s instructions. For a positive control, cells were treated with 5 ng/mL TNF-α during 24 hours. As indicated, cells were treated with 40 ng/mL and 500 ng/mL of sEng. Cells were analyzed 48 hours after transfection and luciferase relative units were determined in a Glomax multidetection system (Promega, Wisconsin, USA). Fold induction was defined as the ratio of relative light units (RLU) produced in treated cells to the RLU produced in untreated cells.

**Quantitative real-time PCR**

For quantitative real-time PCR analysis (qRT-PCR), HUVECs were treated with 40 ng/mL or 500 ng/mL of sEng for 16 hours. Gene expression of *ENG*, *GAPDH*, *VCAM-1*, *ID1*, *SERPINE1*, *NOS3 (eNOS)*, *ACVRL1 (ALK-1)*, *TGFBR1*, *TGFBR2*, *PTGS1 (COX-1)*, *PTGS2 (COX-2)*, *NF-κB*, *CCL2 (MCP-1)* and *STAT3* were examined on 7500 HT Fast Real-Time PCR System (ThermoFisher Scientific), as described previously [37]. Total RNA from HUVECs was isolated using TRI reagent (Sigma-Aldrich) or SpeedTools Total RNA Extraction Kit (Biotools, Florida, USA) according to the manufacturer's protocol and directly converted into cDNA with a high capacity cDNA reverse transcription kit or iScript cDNA Synthesis Kit (ThermoFisher Scientific or BioRad, California, USA, respectively). 30 ng of cDNA was loaded into the reaction and experiments were performed in duplicate. The amplifications were run using TaqMan® Fast Universal PCR Master Mix (ThermoFisher Scientific) and pre-designed TaqMan® Gene Expression Assay kits for the following genes: *ENG* (Hs00923996_m1), *GAPDH* (Hs02758991_g1), *VCAM-1* (Hs01003372_m1), *PTGS2* (Hs00153133_m1), *PTGS1* (Hs00377726_m1), *ID1* (Hs03676575_s1), *SERPINE1* (Hs01126606_m1), *NFKB3* (Hs00153294_m1), *NOS3* (Hs01574659_m1), *CCL2* (Hs00234140_m1), *STAT3* (Hs01047580_m1), *ACVRL1* (Hs00953798), *TGFBR1*

(Hs00610320), *TGFBR2* (Hs00234253_m1) all provided by ThermoFisher Scientific. The time–temperature profile was as follows: 95°C for 3 min; 40 times: 95°C for 7 s, 60°C for 25 s. The relative expression ratio was calculated as described previously [38]. The glyceraldehyde 3-phosphate dehydrogenase gene (*GAPDH*) was used as a reference for normalizing data. Oligonucleotides for IL-6 gene were designed using Roche software for qRT-PCR, human IL-6 Fw 5`- GAAGGCAGCAGGCAACAC-3` and Rv 5`-CAGGAGCCCAGCTATGAACT-3`. As an internal control, mRNA levels of *18S rRNA* were measured using primers Fw 5´-CTCAACACGGGAAACCTCAC-3` and Rv 5`-CGCTCCACCAACTAAGAACG-3`. In this case, amplicons were detected using a LightCycler 480 System II-384 (Roche Applied Biosciences, Switzerland).

**IL-6 ELISA**

HUVEC monolayers were treated with 40 ng/mL or 500 ng/mL of sEng in media without serum. Culture supernatants were collected after 24 hours and analyzed by ELISA. Concentration of human IL-6 in the cell culture media was determined according to the manufacturer protocol by Quantikine Human IL-6 assay (D6050; R&D Systems). The results of the immunoassay were measured in a Glomax Multidetection system (Promega) and normalized by the supernatant protein concentration (µg/mL) of each sample and by the control group levels (100%).

**Western blot analysis**

For detection of protein expression by Western blot analysis, cells were treated with 40 ng/mL or 500 ng/mL of sEng for 2 hours (phospho Smads, peNOS) or 16 hours for the determination of endoglin and 48 hours for the determination of total NF-κB and phosphorylated NF-κB. Samples were homogenized in RIPA buffer (Sigma-Aldrich)

containing protease (SERVA Electrophoresis, Germany) and phosphatase inhibitors (ThermoFisher Scientific). Cell homogenates (20 µg of total protein) were separated as described previously [38]. Membranes were incubated overnight at 4ºC with primary antibodies including anti-human endoglin (sc-20632, Santa Cruz Biotechnology, California, USA) at 1:500 dilution, anti-human peNOS (sc-21871-R, Santa Cruz Biotechnology) at 1:200 dilution, anti-human pSmad1/5 (9516, Cell Signaling, Massachusetts, USA) at 1:500 dilution, anti-human pSmad2/3 (8828, Cell Signaling) at 1:500 dilution, anti-human phospho - NF-κB (ab86299, Abcam, United Kingdom) at 1:2,000 dilution and anti-human NF-κB (ab16502, Abcam) at 1:500 dilution. Equal loading of total proteins was confirmed by immunodetection with anti-human GAPDH (G8795, Sigma-Aldrich) at 1:10,000 dilution. As secondary antibodies, horseradish peroxidase-linked goat anti-rabbit IgG-$(Fab')_2$ (ab6112, Abcam) was used to detect all proteins except GAPDH, at different dilutions: 1:2,000 for anti-endoglin, anti-pSmad1/5, and anti-NF-κB; 1:1,000 for anti-peNOS$^{S1177}$ and anti-pSmad2/3, and 1:4,000 for anti-phospho-NF-κB. For anti-GAPDH detection, the secondary antibody horseradish peroxidase-conjugated goat anti-mouse IgG (Sigma-Aldrich) was used at 1:20,000 dilution. After washing with TBST buffer, membranes were developed using an enhanced chemiluminescent reagent (ThermoFisher Scientific). Quantification of immunoreactive bands on the exposed films was performed by image analysis software NIS (Laboratory Imaging, Czech Republic).

**Immunofluorescence flow cytometry**

For immunofluorescence flow cytometry, HUVECs were treated with 40 ng/mL or 500 ng/mL of sEng for 16 hours in EBM-2 medium. HUVECs were rinsed with PBS, detached with trypsin and then incubated with fluorescein-conjugated mouse monoclonal antibody against human VCAM-1 (CD106; BBA22, R&D Systems) or anti-human endoglin

P4A4 mouse monoclonal antibody (Developmental Studies Hybridoma Bank, University of Iowa, USA) for 1 hour at 4°C. As a negative control for analysis, an irrelevant isotype control antibody IgG2A (IC002F, R&D Systems) was used. The determination of protein expression was performed using the cell staining protocol for flow cytometry on Cell Lab Quanta SC Flow Cytometer (Beckman Coulter, Inc., California, USA). A total number of 5,000 cells was analyzed per sample. Results are presented as relative expression index, calculated as a number of positive cells of the target sample multiplied by the mean fluorescence intensity, related to control samples as described previously [38].

**Statistical analysis**

Results are expressed as mean ± SEM. All multiple comparison data were analyzed using ANOVA with Kruskal-Wallis test followed Dunn's multiple comparisons test and direct group-group comparisons were carried out using the Mann-Whitney test. For the above-mentioned statistical analysis, the GraphPad Prism 6.0 software (GraphPad Software, Inc., California, USA) was used. P values $<0.05$ were considered statistically significant.

**RESULTS**

**Effect of sEng on endothelial dysfunction markers**

Expression analysis by qRT-PCR was used to test whether sEng treatment affects endothelial dysfunction and inflammation markers, such as *VCAM-1, NOS3, NF-κB, STAT3, IL-6, CCL2, PTGS1, and PTGS2* (Fig. 1). Sixteen hours treatment with sEng (40 ng/mL and 500 ng/mL) resulted in a significant increase of *VCAM-1* mRNA expression (only at 500 ng/mL) and *NF-κB, IL-6,* and *PTGS2* in both tested sEng concentrations (Fig. 1). No significant effect of sEng treatment was detected for *NOS3, STAT3, CCL2,* and *PTGS1* (Fig. 1).

In addition, we analyzed significantly upregulated genes on protein level. At the protein level, VCAM-1 analysis using immunofluorescence flow cytometry showed no significant effect upon sEng treatment (Fig. 2). Similarly, protein expression levels of $peNOS^{S1177}$ (endothelial NO synthase phosphorylated at serine 1177, showing increased activity of this enzyme) in total cell extracts showed no significant changes after 2 hours of treatment with 500 ng/mL of sEng under starving conditions, as determined by Western blot analysis (Fig. 2). In the same experiment, no significant changes of COX-2 were detected.

**Fig. 1.** Gene expression of endothelial dysfunction markers. HUVECs were incubated with different concentrations of sEng (40 ng/mL and 500 ng/mL) for 16 hours. Total RNA was subjected to qRT-PCR using probes specific for genes related to endothelial dysfunction and inflammation. mRNA expression of *VCAM-1* (a), *NOS3* (b), *NF-κB* (c), *STAT3* (d), *IL-6* (e), *CCL2* (f), *PTGS1* (g), *PTGS2* (h) are shown. *GAPDH* or *18S rRNA* were used as housekeeping genes to normalize the values. Representative data of six independent experiments with n=3 per experiment are shown. Results are expressed as mean ± SEM. All multiple comparison data were analyzed using ANOVA with Kruskal-Wallis test followed Dunn's multiple comparisons test. *P <0.05, **P <0.01 and ***P <0.001, ns >0.05 versus control samples.

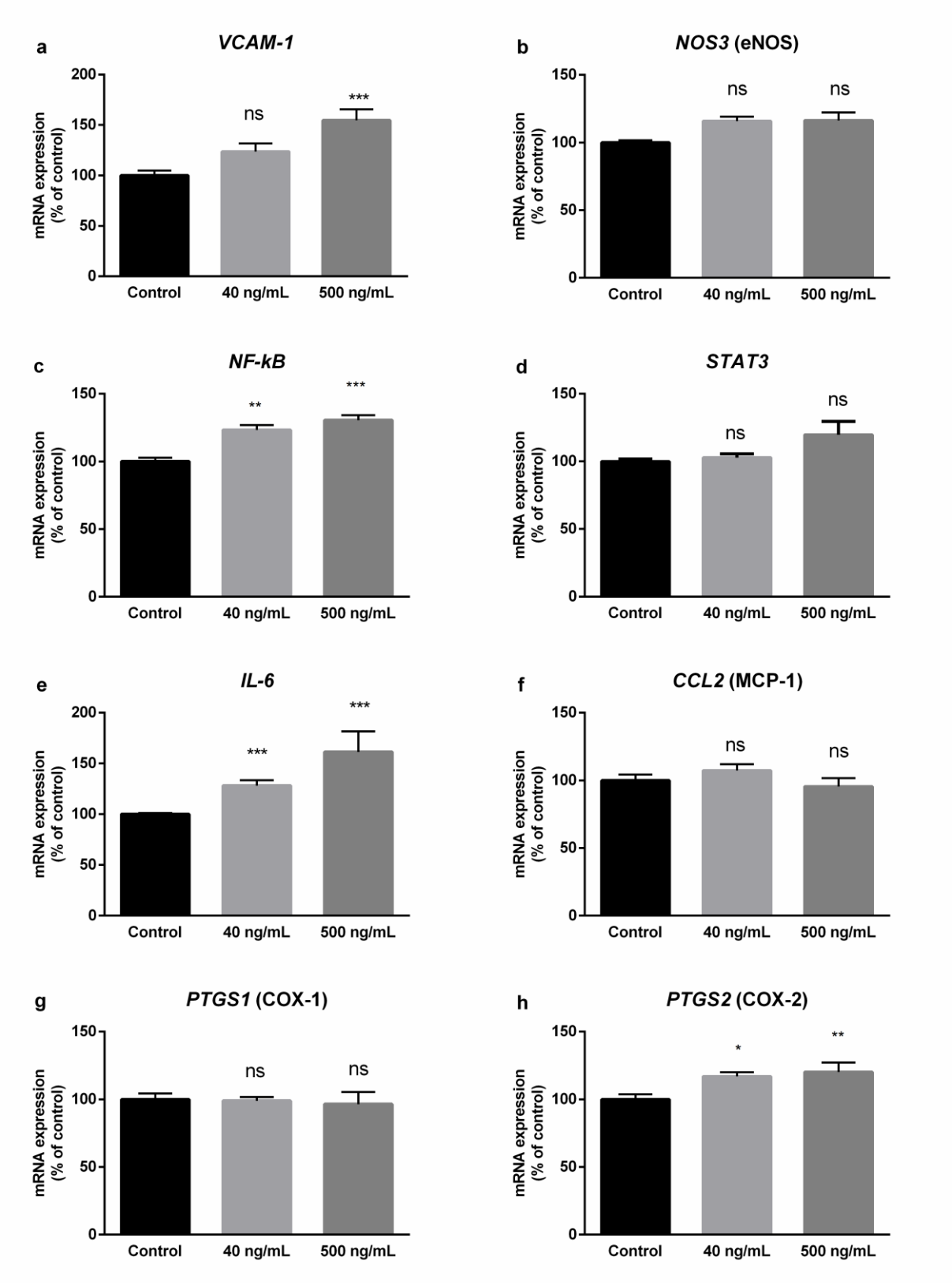
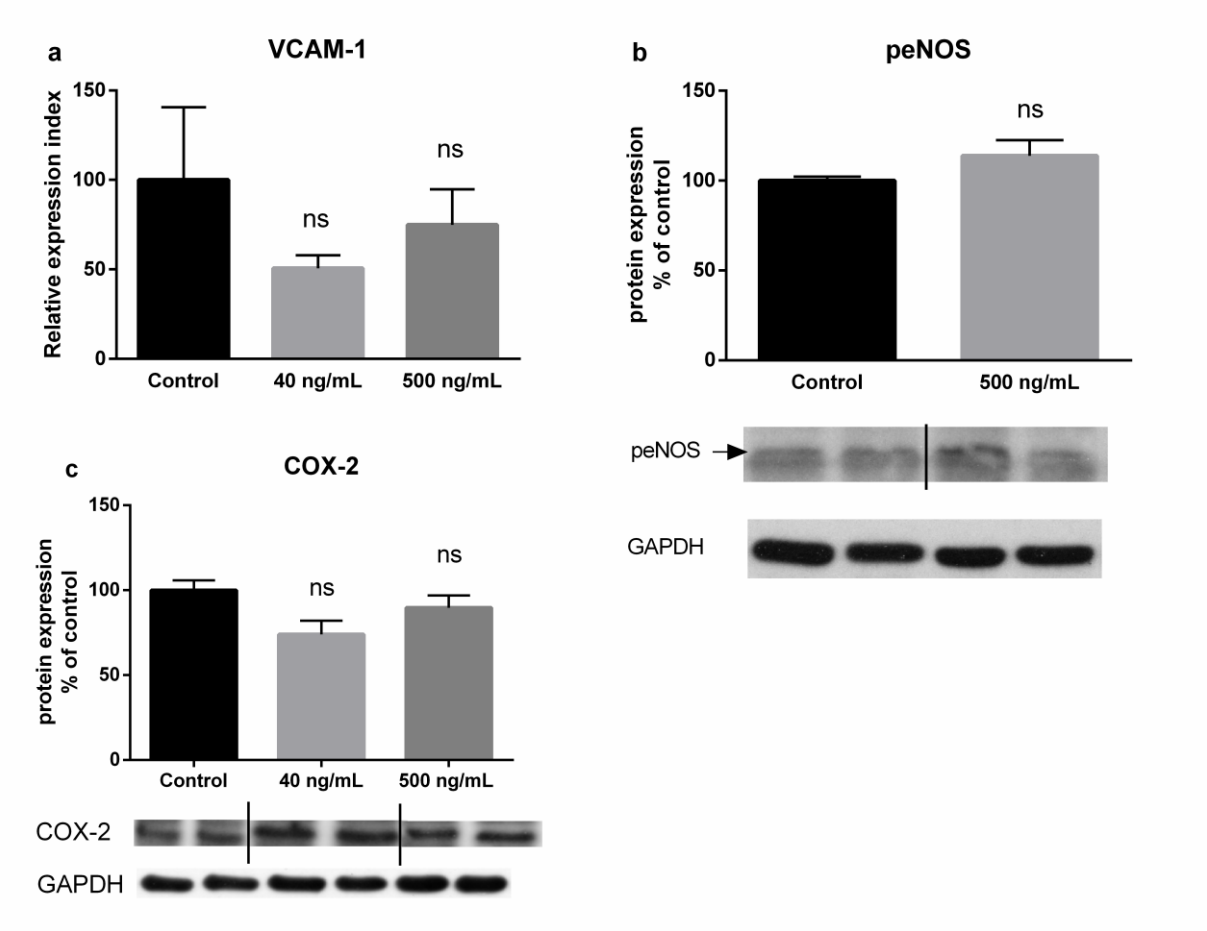


**Fig. 2.** sEng treatment effects on VCAM-1, COX-2 and peNOS$^{S1177}$ expression. For immunofluorescence flow cytometry quantification of VCAM-1 (a), HUVECs were treated with 40 ng/mL or 500 ng/mL of sEng for 16 hours in EBM-2 medium. Results are presented as relative expression index. For Western blot analysis of peNOS$^{S1177}$, HUVECs were treated for 2 hours under starving conditions (b). For Western blot analysis of COX-2, HUVECs were treated for 16 hours (c). The top panel shows the densitometric analysis (control = 100%), whereas the bottom panel includes a representative immunoblot. Equal loading of samples was confirmed by immunodetection of GADPH. Representative data of three independent experiments with n=3 per experiment are shown. Results are expressed as mean ± SEM. All multiple comparison data (VCAM-1, COX-2) were analyzed using ANOVA with Kruskal-Wallis test followed Dunn's multiple comparisons test. Mann-Whitney test we used for the analysis of peNOS. ns >0.05 versus control samples.




**Effect of sEng on components of TGF-β signaling**

To assess, whether sEng had any effect on selected members of TGF-β signaling pathway, we analyzed mRNA expression of *ACVRL1, TGFBR1, TGFBR2, SERPINE1* and *ID1* (Fig. 3). 16 hours treatment with sEng (40 ng/mL and 500 ng/mL) resulted in a significant increase of *ACVRL1 (ALK-1)* and *ID1* (only at 500 ng/mL) mRNA expression (Fig. 3). No significant changes in mRNA expression of *TGFBR1, TGFBR2, SERPINE1* were observed.

Potential activation of TGF-β signaling was assessed by Western blot analysis of pSmad2/3 and pSmad1/5 protein expression (Fig. 4). The results showed a significant increase of pSmad2/3 protein expression after 2 hours of treatment with 500 ng/mL sEng (Fig. 4), but no significant change in the protein expression of pSmad1/5 was observed when

compared to control (Fig. 4). In addition, we aimed to evaluate protein levels of Id-1, whose gene expression was increased upon treatment with sEng (500 ng/mL) for 16h (Fig. 3). Surprisingly Id-1 expression was significantly lower after 500 ng/mL of sEng treatment when compared to control (Fig. 4).

**Fig. 3.** Gene expression of *ACVRL1* (a), *TGFBR1* (b), *TGFBR2* (c), *SERPINE1* (d) and *ID1* (e). HUVECs were incubated with different concentrations of sEng (40 ng/mL and 500 ng/mL) for 16 hours. Representative data of six independent experiments with n=3 per experiment are shown. Results are expressed as mean ± SEM. All multiple comparison data were analyzed using ANOVA with Kruskal-Wallis test followed Dunn's multiple comparisons test. *P <0.05 and ***P <0.001, ns >0.05 versus control samples.

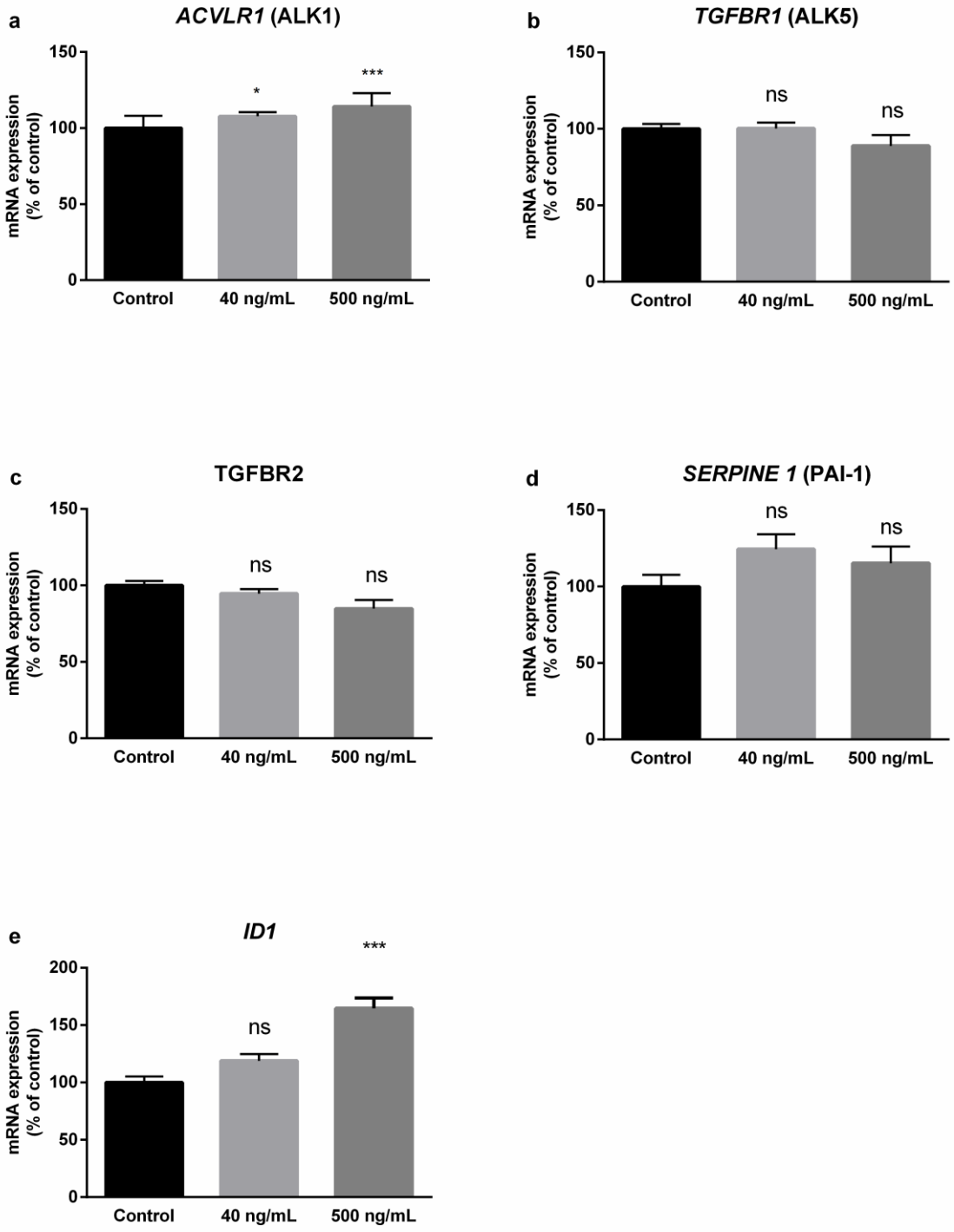
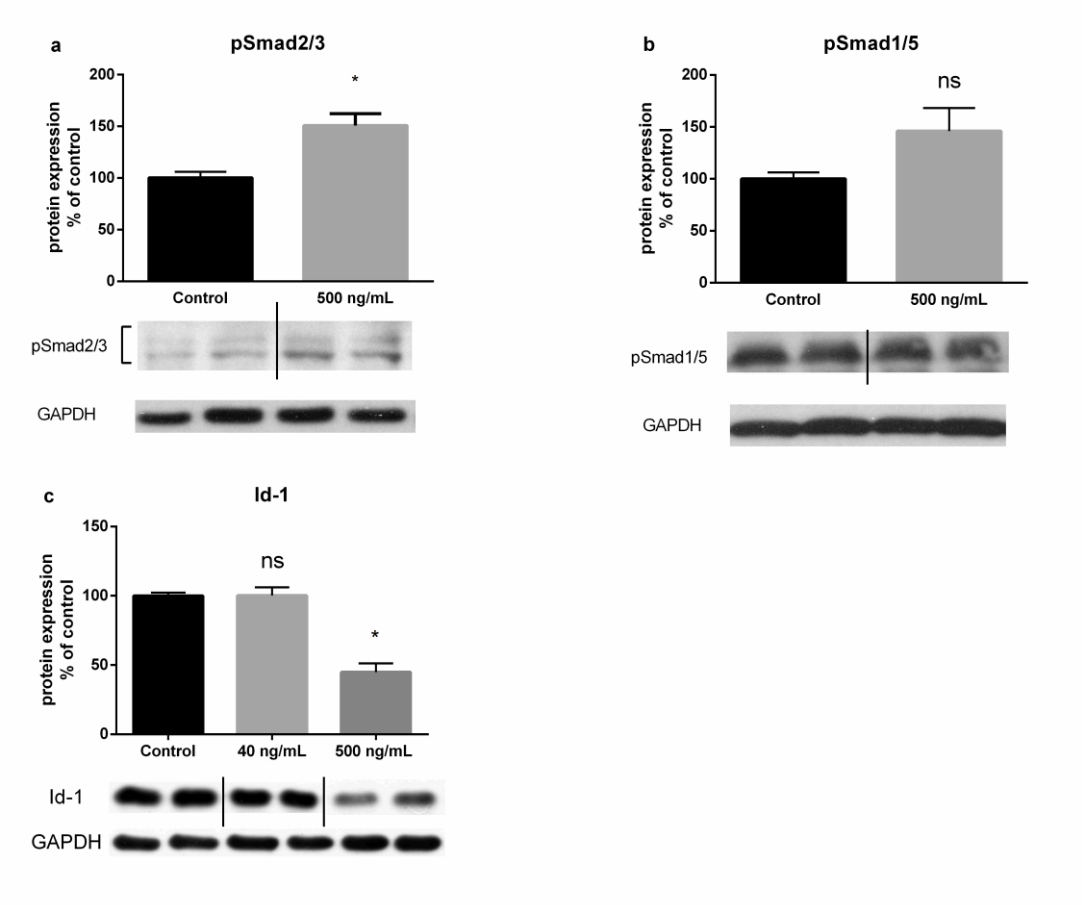


**Fig. 4.** Protein expression levels of pSmad2/3 (a), pSmad1/5 (b) analyzed by Western blot after 2 hours of treatment with 40 ng/mL or 500 ng/mL sEng under the starving conditions. Id-1 expression (c) analyzed by Western blot after 16 hours of treatment with 40 ng/mL or 500 ng/mL sEng. Presented data show one representative of three independent experiments with n=3 per experiment for Western blot analysis. A densitometric analysis (control = 100%) has been done and equal loading of samples was confirmed by immunodetection of GADPH. Results are expressed as mean ± SEM. All multiple comparison data (Id-1) were analyzed using ANOVA with Kruskal-Wallis test followed Dunn's multiple comparisons test. Mann-Whitney test we used for the analysis of pSmad2/3 and pSmad1/5. *P <0.05, ns >0.05 versus control samples.

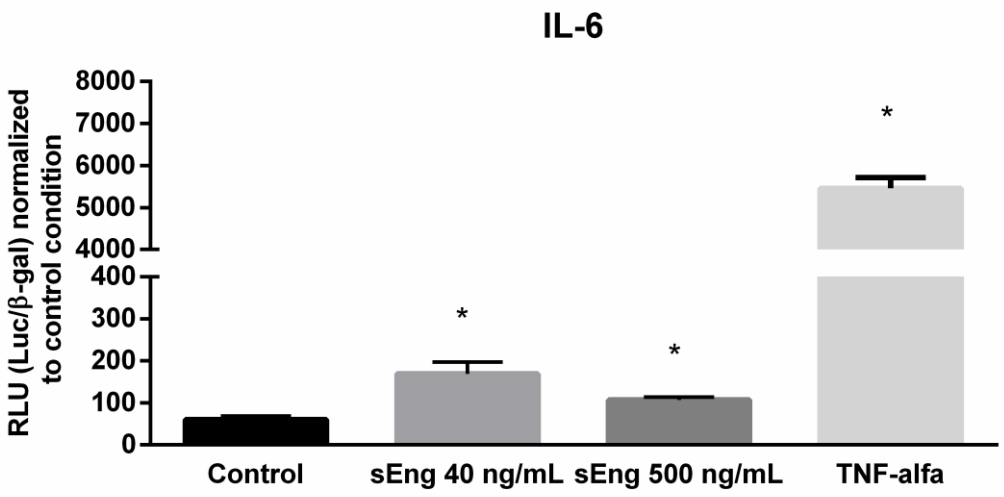


**sEng treatment activates IL-6 promoter/gene reporter and IL-6 protein levels.**

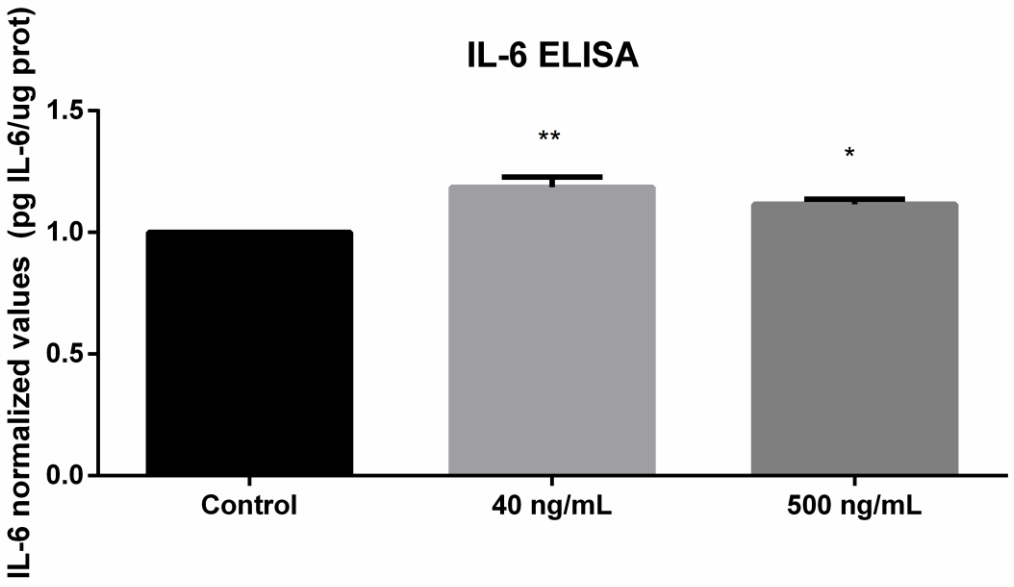
Since significant differences in mRNA expression of *IL-6* were detected, we wanted to test if sEng is able to stimulate the transcriptional activity of IL-6 (Fig. 5). The IL6 reporter vector encodes the luciferase driven by the IL6 promoter. Transient transfection experiments were performed in HEK293T cells with luciferase-IL-6-promoter (pGL3-IL-6-Luc). There is a clear marked activation of the IL-6 promoter vector due to sEng treatment (sEng: 40 ng/mL and 500 ng/mL for 24 hours). These results suggest that sEng is able to stimulate the transcriptional activity of IL-6 (Fig. 5). In addition, we confirmed a significant increase of IL-6 protein levels in cultured media after 24 hours incubation with 40 ng/mL and 500 ng/mL of sEng in HUVECs.

**Fig. 5.** IL-6 luciferase reporter expression in HEK293T cells transfected with pGL3-luc-IL-6 vector. sEng effects on the activity of the IL-6-pGL3-luciferase promoter after 24h of treatment with sEng 40 ng/mL and 500 ng/mL (a). Results are normalized between empty-luc promoter vector pGL3 and expressed as a fold induction with respect to the basal activity of the IL-6-pGL3-luciferase promoter vector activity without treatment. Transfection efficiency was corrected by relating luciferase to β-galactosidase activity. This is one representative experiment of three independent ones. In the same experiment, TNFα was used as a positive control. HUVECs were treated with 40 ng/mL or 500 ng/mL sEng for 24 hours and protein levels of IL6 were measured by ELISA (b). IL6 protein levels were normalized for protein concentration and compared to those of untreated cells that were given an arbitrary value of 1. Representative data of three independent experiments with n=3 per experiment are shown. Results are expressed as mean ± SEM. All multiple comparison data were analyzed using ANOVA with Kruskal-Wallis test followed Dunn's multiple comparisons test. *P <0.05 and **P <0.01, versus control samples.

a




b

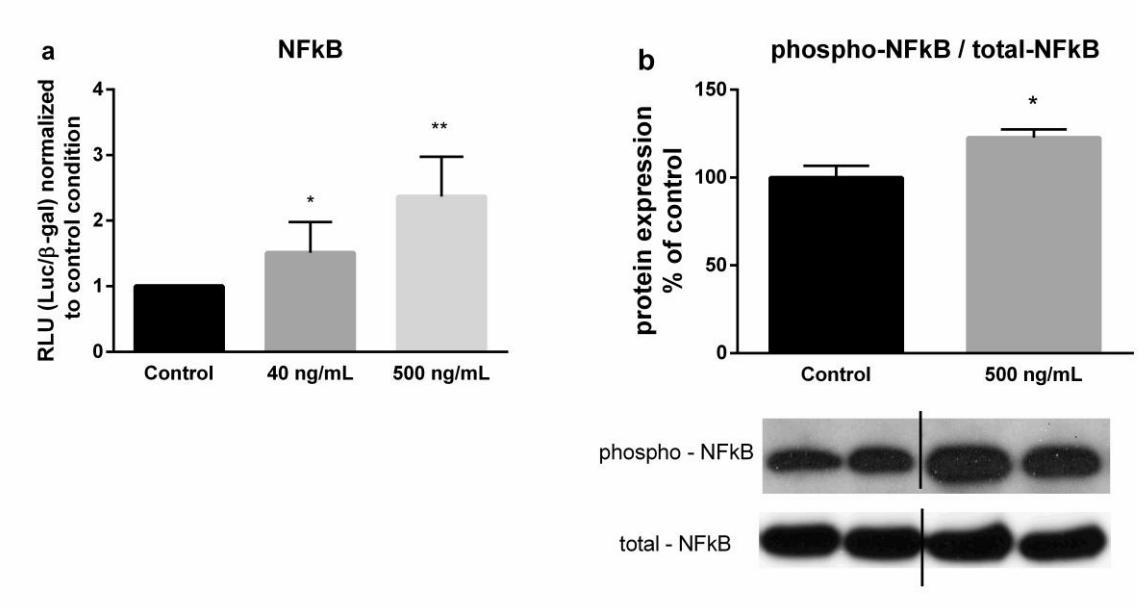


**sEng treatment activates NF-κB signaling and expression**

HEK293T cells and HUVECs were used to evaluate whether sEng affects the pro-inflammatory activity, expression and phosphorylation of NF-κB. In order to evaluate sEng effects on transcriptional activity of NF-κB, HEK293 cells were transfected with pGL2-KBF-luciferase reporter vector and incubated for 24 hours with increasing concentrations of sEng: 40 ng/mL, and 500 ng/mL. The NF-κB reporter encodes the luciferase driven by a regulatory sequence containing consensus motifs for NF-κB binding and therefore is responsive to NF-κB activation. Figure 6 shows an increment of pGL2-KBF-luc vector when compared to the basal levels of pGL2-KBF-Luciferase vector without treatment. To elucidate whether sEng affects NF-κB expression and phosphorylation status, a Western blot analysis was performed and phospho/total NF-κB ratio of intensities was determined. The results showed increased phosphorylation of NF-κB in HUVECs treated for 48 hours with 500 ng/mL of sEng (Fig. 6).

**Fig. 6.** NF-κB expression and activity in HEK293 cells and HUVECs. HEK293T cells were transfected with pGL2-KBF-luciferase reporter vector and incubated for 24 hours with 40 ng/mL, 100 ng/mL or 500 ng/mL of sEng (a). Results are represented as fold-induction values with respect to the activity of the untreated sample. This is one representative experiment of three independent ones. Protein expression of total and phospho-NF-κB (b). HUVECs were incubated in the absence or presence of 500 ng/mL of sEng for 48 hours. Total protein extracts were subjected to Western blot analysis using specific antibody to total and phospho-NF-κB. Results shown are representative of three independent experiments with n=3 per experiment. The top panel represents the densitometric analysis (control=100%). The bottom panel includes a representative immunoblot. Results are expressed as mean ± SEM. All multiple comparison data were analyzed using ANOVA with Kruskal-Wallis test followed Dunn's multiple comparisons test. Mann-Whitney test was used for the statistical analysis of

phospho/total NF-κB ratio of intensities by Western blot. *P <0.05, **P <0.01 and ns >0.05 versus control samples.

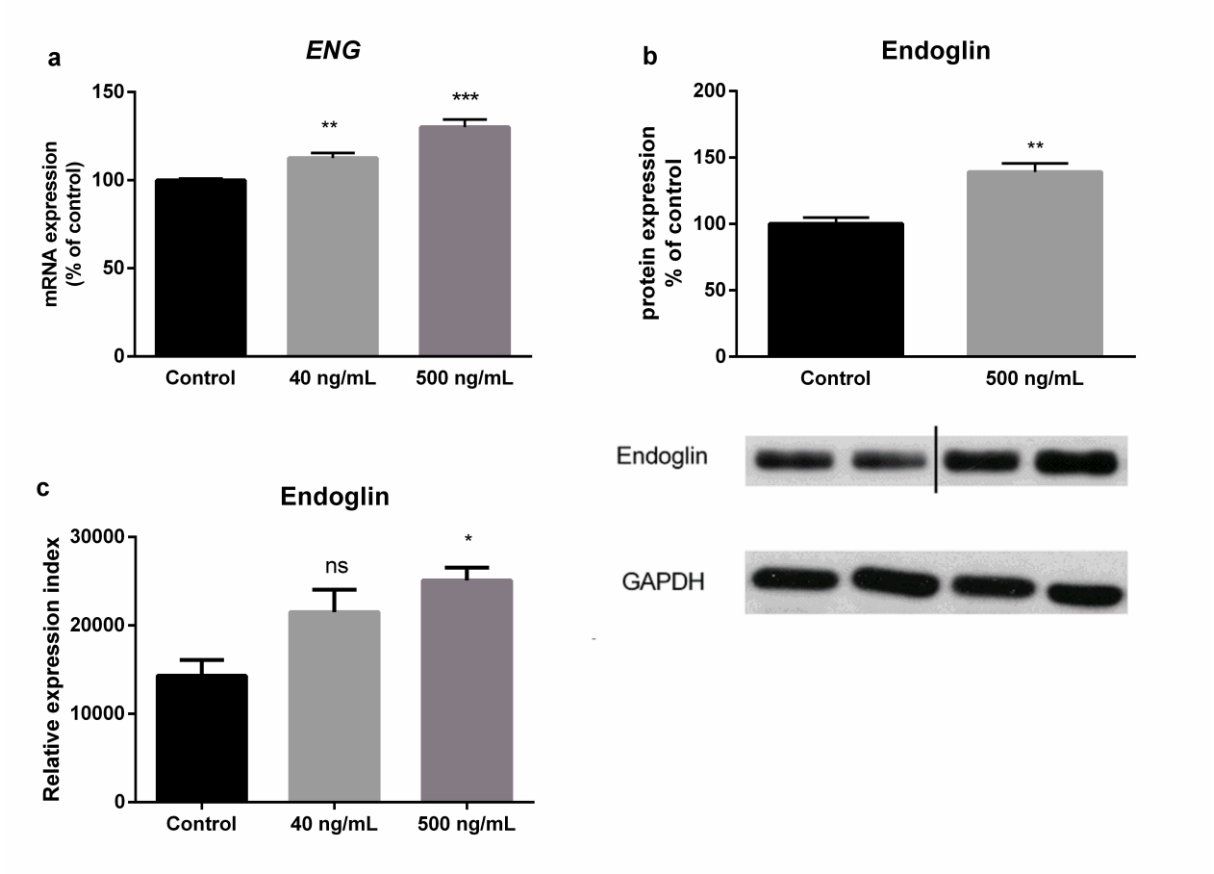



**sEng treatment increases membrane endoglin expression in HUVECs**

To assess whether sEng can cause changes in mRNA and protein expression level of membrane endoglin, qRT-PCR and Western blot analyses were performed. Total RNA was subjected to qRT-PCR using probes specific for human endoglin. A significant upregulation of mRNA of endoglin is observed at the highest concentration of sEng (Fig. 7). In addition, Flow cytometry and Western blot analysis confirmed significant increase of the protein level after the treatment with 500 ng/mL of sEng (Fig. 7).

**Fig. 7. Endoglin expression levels after sEng treatment.** Expression levels of human endoglin mRNA after 16 hours of treatment with 40 ng/mL and 500 ng/mL of sEng, as determined by qRT-PCR analysis (a). Protein expression levels of membrane endoglin after 16 hours of treatment with 500 ng/mL of sEng (b). Protein expression levels of membrane endoglin after 16 hours of treatment with 40 ng/mL and 500 ng/mL of sEng, as determined by flow cytometry (c). Total protein extracts were subjected to Western blot analysis using

specific antibody to endoglin. The top panel shows the densitometric analysis (control = 100%), whereas the bottom panel includes a representative immunoblot. Equal loading of samples was confirmed by immunodetection of GADPH. Flow cytometry results are presented as relative expression index. Representative data of three independent experiments with n=3 per experiment are shown. Results are expressed as mean ± SEM. All multiple comparison data were analyzed using ANOVA with Kruskal-Wallis test followed Dunn's multiple comparisons test. Mann-Whitney test was used for the statistical analysis of endoglin expression by Western blot analysis. *P <0.05, **P <0.01 and ***P <0.001, ns >0.05 versus control samples.



## DISCUSSION

The results of this study show that sEng treatment results in activation of NF-κB/IL-6 expression suggesting that sEng treatment is responsible for the partial development of pro-inflammatory phenotype in HUVECs.

Increased levels of sEng are released from membrane bound endoglin into the circulation in different settings of cardiovascular pathophysiology, including hypercholesterolemia [1], hypertension [34], inflammation [21], atherosclerosis [39], endothelial injury [40], type II diabetes mellitus [9] and preeclampsia [17]. In addition to being a potential biomarker of these pathologies, the interaction of sEng with various members of TGF-β resulting in manifestation of endothelium inflammation/alteration/dysfunction has been postulated [32,41,42]. Therefore, in this study, we hypothesized that high levels of sEng may contribute to inflammation/endothelial dysfunction of cultured human endothelial cells.

A previous report has shown that sEng interacts with TGF-β/Smad2/3 signaling pathway, leading to the inhibition of eNOS-dependent vasodilatation in isolated rat renal microvessels and mesenteric vessels [41]. In addition, adenovirus-driven expression of sEng resulted in neutralization of TGF-β and VEGF effects with a subsequent increase in expression of P-selectin and leukocyte rolling to endothelium, elevated levels of soluble E-selectin, soluble VCAM-1 and impaired vasodilatation [42]. sEng can scavenge BMP-9 and block BMP-9-mediated Smad1/5/8 phosphorylation [17]. These background data prompted us to evaluate whether markers of endothelial inflammation/dysfunction were affected by sEng treatment at 40 ng/mL and 500 ng/mL concentration. The dose 40 ng/mL is closely related to levels of sEng measured in patient with preeclampsia [43], whereas 500 ng/mL dose was used as a proof of concept of our hypothesis and is related to our previous *in vivo* experiments [32,33].

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Endothelial dysfunction is primarily related to the functional changes involving NO production and alteration of eNOS-dependent vasodilatation [44]. In addition, increased expression of cell adhesion molecules and other pro-inflammatory molecules, including MCP-1, COX1, and COX2 was proposed to be involved in the manifestation of endothelial dysfunction and inflammation, which is a crucial step in the development of many cardiovascular diseases, such as atherosclerosis or hypertension [22,23,45]. Even though the above-mentioned biomarkers were not significantly affected by sEng on protein levels, we found that sEng treatment activates and increases gene expression and phosphorylation status of NF-κB (reflecting increased protein activity), which is a key inflammatory transcriptional factor. Moreover, NF-κB was demonstrated to activate the expression of many pro-inflammatory molecules, including IL-6 [30]. Accordingly, in our study sEng treatment activated IL-6 expression. NF-κB and IL-6 were demonstrated to be related to the development of endothelial dysfunction and acute inflammation [46]. However, the fact that other NF-κB-regulated pro-inflammatory proteins were not affected by sEng suggests that sEng treatment by itself does not induce a complete inflammatory status in endothelial cells in our experimental design.

Surprisingly, sEng treatment also resulted in an increased expression of membrane bound endoglin. Possible explanation of this increase might be related to the fact that the effect of soluble endoglin is related to the increase of Smad2/Smad3 phosphorylation, which is a downstream target of the TGF-beta signaling pathway. Supporting this interpretation is the fact that it has been shown that TGF-beta markedly enhances the expression of cell surface endoglin [47]. Membrane bound endoglin was suggested to potentiate eNOS activity, stability and expression via Smad2 signaling, which is important for the maintenance of physiological properties of endothelium *in vivo* [13]. On the other hand, membrane bound endoglin was proposed to be involved in transmigration of leukocytes during inflammatory reaction [21].

Therefore, it is tempting to speculate that increased expression of endoglin, together with activation of NF-κB/IL-6 signaling, could be, at least partially, related to induced inflammation after sEng treatment in endothelial cells [48].

Because endoglin is a component of the TGF-β receptor complex, we analyzed the expression levels of some members of the TGF-β signaling pathway in order to evaluate a possible impact of increased endoglin expression. Previous studies demonstrated that endoglin modulates the TGFβRII/ALK-1/Smad1/5/ld1 and TGFβRII/ALK-5/Smad2/3/PAI-1 pathways [2]. However, in our study, expression levels of members of these signaling cascades were not affected by sEng, except for increased pSmad2/3 and decreased Id-1 expression. Intriguingly, Smad2/3 phosphorylation was induced by sEng without affecting the expression of the downstream target PAI-1, suggesting that this pathway is not fully activated after sEng treatment. Moreover, we found no changes of eNOS ($Ser^{1177}$) phosphorylation in HUVECs as well, suggesting that endoglin/eNOS pathway is not activated as well. Id-1 is a transcription factor participating on TGF-β signaling, however its precise role in inflammation is unknown. Protein levels of Id1 were reduced upon 500 ng/mL sEng treatment, although under the same conditions Id-1 transcripts were increased. The reasons for this discrepancy are unclear, but the existence of additional regulatory mechanisms such as post-transcriptional and post-translational modifications may probably account for these differences. Of note, protein expression is expected to be more relevant than transcript levels with respect to the function of this transcription factor. We propose that reduced Id1 protein expression might be related to the fact that soluble endoglin has been postulated to antagonize the function of membrane endoglin [10,16]. Because membrane endoglin contributes together with ALK1/Smad1,5,8 to a signaling pathway that leads to an upregulation of Id1, then it would be expected that soluble endoglin downregulates Id1. This would be compatible with the increased phosphorylation of Smad 2,3 because ALK5 signaling is favored when ALK1/endoglin

signaling is inhibited. However, clear impact of reduced Id-1 expression on NF-κB and IL-6 expression remains to be elucidated. In general, the precise consequences of increased endoglin expression after sEng treatment will be addressed in our prospective studies.

Since sEng treatment only induces a partial inflammatory reaction in endothelial cells, we speculate that this could be related to the fact that some additional stimuli are needed. Although sEng is responsible for an increased arterial pressure in mice [34], the presence of high levels of sEng did not induce signs of endothelial dysfunction in mouse aorta [33]. However, it is of interest to mention that high levels of sEng will always be present in the circulation together with other possible risk factors of cardiovascular disease, including hypercholesterolemia or hyperglycemia. Indeed, we demonstrated very recently that a combination of a mild hypercholesterolemia and high levels of soluble endoglin increases markers of endothelial inflammation, including NF-κB, in mouse aorta [32]. Thus, we propose that sEng may act more substantially in collaboration with other potential cardiovascular risk factors (oxLDL, glucose, or other circulating cells e.g. leukocytes or mural cells). Further studies on the complex impact of sEng in endothelial cells are currently being addressed in our laboratory.

## CONCLUSION

We report here for the first time that sEng treatment of endothelial cells results in an activation of the pro-inflammatory biomarkers NF-κB and IL-6, suggesting a potential contribution to the pro-inflammatory phenotype in endothelium. The precise mechanism of this activation and its consequence remains to be elucidated. Future studies on the combined action of sEng with other cardiovascular risk factors may help to better understand the role of sEng not only as a biomarker of cardiovascular diseases, but also as a contributor to endothelial dysfunction.

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**CONFLICT OF INTEREST**

The authors declare they have no potential conflict of interest.

**REFERENCES**

1. Rathouska J, Jezkova K, Nemeckova I, Nachtigal P (2015) Soluble endoglin, hypercholesterolemia and endothelial dysfunction. Atherosclerosis 243: 383-388.

2. Lopez-Novoa JM, Bernabeu C (2010) The physiological role of endoglin in the cardiovascular system. Am J Physiol Heart Circ Physiol 299: H959-974.

3. Cheifetz S, Bellon T, Cales C, Vera S, Bernabeu C, Massague J, Letarte M (1992) Endoglin is a component of the transforming growth factor-beta receptor system in human endothelial cells. J Biol Chem 267: 19027-19030.

4. Lastres P, Martin-Perez J, Langa C, Bernabeu C (1994) Phosphorylation of the human-transforming-growth-factor-beta-binding protein endoglin. Biochem J 301 ( Pt 3): 765-768.

5. Conley BA, Smith JD, Guerrero-Esteo M, Bernabeu C, Vary CP (2000) Endoglin, a TGF-beta receptor-associated protein, is expressed by smooth muscle cells in human atherosclerotic plaques. Atherosclerosis 153: 323-335.

6. Nachtigal P, Zemankova Vecerova L, Rathouska J, Strasky Z (2012) The role of endoglin in atherosclerosis. Atherosclerosis 224: 4-11.

7. Hawinkels LJ, Kuiper P, Wiercinska E, Verspaget HW, Liu Z, Pardali E, Sier CF, ten Dijke P (2010) Matrix metalloproteinase-14 (MT1-MMP)-mediated endoglin shedding inhibits tumor angiogenesis. Cancer Res 70: 4141-4150.

8. Ikemoto T, Hojo Y, Kondo H, Takahashi N, Hirose M, Nishimura Y, Katsuki T, Shimada K, Kario K (2012) Plasma endoglin as a marker to predict cardiovascular events in patients with chronic coronary artery diseases. Heart Vessels 27: 344-351.

9. Blazquez-Medela AM, Garcia-Ortiz L, Gomez-Marcos MA, Recio-Rodriguez JI, Sanchez-Rodriguez A, Lopez-Novoa JM, Martinez-Salgado C (2010) Increased plasma soluble

endoglin levels as an indicator of cardiovascular alterations in hypertensive and diabetic patients. BMC Med 8: 86.

10. Oujo B, Perez-Barriocanal F, Bernabeu C, Lopez-Novoa JM (2013) Membrane and soluble forms of endoglin in preeclampsia. Curr Mol Med 13: 1345-1357.
11. Santibanez JF, Quintanilla M, Bernabeu C (2011) TGF-beta/TGF-beta receptor system and its role in physiological and pathological conditions. Clin Sci (Lond) 121: 233-251.
12. Tian F, Zhou AX, Smits AM, Larsson E, Goumans MJ, Heldin CH, Boren J, Akyurek LM (2010) Endothelial cells are activated during hypoxia via endoglin/ALK-1/SMAD1/5 signaling in vivo and in vitro. Biochem Biophys Res Commun 392: 283-288.
13. Santibanez JF, Letamendia A, Perez-Barriocanal F, Silvestri C, Saura M, Vary CP, Lopez-Novoa JM, Attisano L, Bernabeu C (2007) Endoglin increases eNOS expression by modulating Smad2 protein levels and Smad2-dependent TGF-beta signaling. J Cell Physiol 210: 456-468.
14. ten Dijke P, Goumans MJ, Pardali E (2008) Endoglin in angiogenesis and vascular diseases. Angiogenesis 11: 79-89.
15. Tian F, Zhou, A.X., Smits, A.M., et al. (2010) Endothelial cells are activated during hypoxia via endoglin/ALK-1/Smad1/5 signaling in vitro. Biochem Biophys Res Commun. pp. 283-288.
16. Goumans MJ, Liu Z, ten Dijke P (2009) TGF-beta signaling in vascular biology and dysfunction. Cell Res 19: 116-127.
17. Gregory AL, Xu G, Sotov V, Letarte M (2014) Review: the enigmatic role of endoglin in the placenta. Placenta 35 Suppl: S93-99.

18. Toporsian M, Gros R, Kabir MG, Vera S, Govindaraju K, Eidelman DH, Husain M, Letarte M (2005) A role for endoglin in coupling eNOS activity and regulating vascular tone revealed in hereditary hemorrhagic telangiectasia. Circ Res 96: 684-692.

19. Chatterjee A, Black SM, Catravas JD (2008) Endothelial nitric oxide (NO) and its pathophysiologic regulation. Vascul Pharmacol 49: 134-140.

20. Ponnuswamy P, Schrottle A, Ostermeier E, Gruner S, Huang PL, Ertl G, Hoffmann U, Nieswandt B, Kuhlencordt PJ (2012) eNOS protects from atherosclerosis despite relevant superoxide production by the enzyme in apoE mice. PLoS One 7: e30193.

21. Rossi E, Sanz-Rodriguez F, Eleno N, Duwell A, Blanco FJ, Langa C, Botella LM, Cabanas C, Lopez-Novoa JM, Bernabeu C (2013) Endothelial endoglin is involved in inflammation: role in leukocyte adhesion and transmigration. Blood 121: 403-415.

22. Bhaskar S, Sudhakaran PR, Helen A (2016) Quercetin attenuates atherosclerotic inflammation and adhesion molecule expression by modulating TLR-NF-kappaB signaling pathway. Cell Immunol 310: 131-140.

23. Feletou M, Huang Y, Vanhoutte PM (2011) Endothelium-mediated control of vascular tone: COX-1 and COX-2 products. Br J Pharmacol 164: 894-912.

24. Brasier AR (2010) The nuclear factor-kappaB-interleukin-6 signalling pathway mediating vascular inflammation. Cardiovasc Res 86: 211-218.

25. Xia YF, Liu LP, Zhong CP, Geng JG (2001) NF-kappaB activation for constitutive expression of VCAM-1 and ICAM-1 on B lymphocytes and plasma cells. Biochem Biophys Res Commun 289: 851-856.

26. Hoesel B, Schmid JA (2013) The complexity of NF-kappaB signaling in inflammation and cancer. Mol Cancer 12: 86.

27. Karin M, Lin A (2002) NF-kappaB at the crossroads of life and death. Nat Immunol 3: 221-227.

28. Gingery A, Bradley EW, Pederson L, Ruan M, Horwood NJ, Oursler MJ (2008) TGF-beta coordinately activates TAK1/MEK/AKT/NFkB and SMAD pathways to promote osteoclast survival. Exp Cell Res 314: 2725-2738.

29. Tobar N, Villar V, Santibanez JF (2010) ROS-NFkappaB mediates TGF-beta1-induced expression of urokinase-type plasminogen activator, matrix metalloproteinase-9 and cell invasion. Mol Cell Biochem 340: 195-202.

30. Libermann TA, Baltimore D (1990) Activation of interleukin-6 gene expression through the NF-kappa B transcription factor. Mol Cell Biol 10: 2327-2334.

31. Simpson RJ, Hammacher A, Smith DK, Matthews JM, Ward LD (1997) Interleukin-6: structure-function relationships. Protein Sci 6: 929-955.

32. Jezkova K, Rathouska J, Nemeckova I, Fikrova P, Dolezelova E, Varejckova M, Vitverova B, Tysonova K, Serwadczak A, Buczek E, Bernabeu C, Lopez-Novoa JM, Chlopicki S, Nachtigal P (2016) High Levels of Soluble Endoglin Induce a Proinflammatory and Oxidative-Stress Phenotype Associated with Preserved NO-Dependent Vasodilatation in Aortas from Mice Fed a High-Fat Diet. J Vasc Res 53: 149-162.

33. Nemeckova I, Serwadczak A, Oujo B, Jezkova K, Rathouska J, Fikrova P, Varejckova M, Bernabeu C, Lopez-Novoa JM, Chlopicki S, Nachtigal P (2015) High Soluble Endoglin Levels Do Not Induce Endothelial Dysfunction in Mouse Aorta. PLoS One 10: e0119665.

34. Valbuena-Diez AC, Blanco FJ, Oujo B, Langa C, Gonzalez-Nunez M, Llano E, Pendas AM, Diaz M, Castrillo A, Lopez-Novoa JM, Bernabeu C (2012) Oxysterol-induced soluble endoglin release and its involvement in hypertension. Circulation 126: 2612-2624.

35. Yano O, Kanellopoulos J, Kieran M, Le Bail O, Israel A, Kourilsky P (1987) Purification of KBF1, a common factor binding to both H-2 and beta 2-microglobulin enhancers. EMBO J 6: 3317-3324.

36. Garrido-Martin EM, Blanco FJ, Roque M, Novensa L, Tarocchi M, Lang UE, Suzuki T, Friedman SL, Botella LM, Bernabeu C (2013) Vascular injury triggers Kruppel-like factor 6 mobilization and cooperation with specificity protein 1 to promote endothelial activation through upregulation of the activin receptor-like kinase 1 gene. Circ Res 112: 113-127.

37. Brcakova E, Fuksa L, Cermanova J, Kolouchova G, Hroch M, Hirsova P, Martinkova J, Staud F, Micuda S (2009) Alteration of methotrexate biliary and renal elimination during extrahepatic and intrahepatic cholestasis in rats. Biol Pharm Bull 32: 1978-1985.

38. Zemankova L, Varejckova M, Dolezalova E, Fikrova P, Jezkova K, Rathouska J, Cerveny L, Botella LM, Bernabeu C, Nemeckova I, Nachtigal P (2015) Atorvastatin-induced endothelial nitric oxide synthase expression in endothelial cells is mediated by endoglin. J Physiol Pharmacol 66: 403-413.

39. Jang YS, Choi IH (2014) Contrasting roles of different endoglin forms in atherosclerosis. Immune Netw 14: 237-240.

40. Gallardo-Vara E, Blanco FJ, Roque M, Friedman SL, Suzuki T, Botella LM, Bernabeu C (2016) Transcription factor KLF6 upregulates expression of metalloprotease MMP14 and subsequent release of soluble endoglin during vascular injury. Angiogenesis 19: 155-171.

41. Venkatesha S, Toporsian M, Lam C, Hanai J, Mammoto T, Kim YM, Bdolah Y, Lim KH, Yuan HT, Libermann TA, Stillman IE, Roberts D, D'Amore PA, Epstein FH, Sellke

FW, Romero R, Sukhatme VP, Letarte M, Karumanchi SA (2006) Soluble endoglin contributes to the pathogenesis of preeclampsia. Nat Med 12: 642-649.

42. Walshe TE, Dole VS, Maharaj AS, Patten IS, Wagner DD, D'Amore PA (2009) Inhibition of VEGF or TGF-{beta} signaling activates endothelium and increases leukocyte rolling. Arterioscler Thromb Vasc Biol 29: 1185-1192.

43. Hirashima C, Ohkuchi A, Matsubara S, Suzuki H, Takahashi K, Usui R, Suzuki M (2008) Alteration of serum soluble endoglin levels after the onset of preeclampsia is more pronounced in women with early-onset. Hypertens Res 31: 1541-1548.

44. Davignon J, Ganz P (2004) Role of endothelial dysfunction in atherosclerosis. Circulation 109: III27-32.

45. Santos-Gallego CG, Picatoste B, Badimon JJ (2014) Pathophysiology of acute coronary syndrome. Curr Atheroscler Rep 16: 401.

46. Aggarwal BB, Gehlot P (2009) Inflammation and cancer: how friendly is the relationship for cancer patients? Curr Opin Pharmacol 9: 351-369.

47. Lastres P, Letamendia A, Zhang H, Rius C, Almendro N, Raab U, Lopez LA, Langa C, Fabra A, Letarte M, Bernabeu C (1996) Endoglin modulates cellular responses to TGF-beta 1. J Cell Biol 133: 1109-1121.

48. Mejia-Rangel J, Cordova E, Orozco L, Ventura-Gallegos JL, Mitre-Aguilar I, Escalona-Guzman A, Vadillo F, Vazquez-Prado J, Gariglio P, Zentella-Dehesa A (2016) Pro-adhesive phenotype of normal endothelial cells responding to metastatic breast cancer cell conditioned medium is linked to NFkappaB-mediated transcriptomic regulation. Int J Oncol 49: 2173-2185.